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Apoptosis and Neurologic Disease

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Many neurological disorders involve cell death. During development of the nervous system, cell death is a normal feature. Elimination of substantial numbers of initially generated cells enables useful pruning of "mismatched" or excessive cells produced by exuberance during the proliferative and migratory phases of development. Such cell death, occurring by "programmed" pathways, is termed apoptosis. In mature organisms, cells die in two major fashions, either by necrosis or apoptosis. In the adult nervous system, because there is little cell production during adulthood, there is little normal cell death. However, neurological disease is often associated with significant neural cell death. Acute disorders, occurring over minutes to hours, such as brain trauma, infarction, hemorrhage, or infection, prominently involve cell death, much of which is by necrosis. Chronic disorders, with relatively slow central nervous system degeneration, may occur over years or decades, but may involve cell losses. Such disorders include motor neuron diseases such as amyotrophic lateral sclerosis (ALS), cerebral dementing disorders such as Alzheimer's disease and frontotemporal dementia, and a variety of degenerative movement disorders including Parkinson's disease, Huntington's disease, and the inherited ataxias. There is evidence that the mechanism of neuronal cell death in these disorders may involve apoptosis. Direct conclusive evidence of apoptosis is scarce in these chronic disorders, because of the swiftness of cell death in relation to the slowness of the disease. Thus, at any particular time point of assessment, very few cells would be expected to be undergoing death. However, it is clearly of importance to define the type of cell death in these disorders. Of significance is that while treating the underlying causes of these conditions is an admirable goal, it may also be possible to develop productive therapies based on alleviating the process of cell death. This is particularly likely if this cell loss is through apoptosis, a programmed process for which the molecular cascade is increasingly understood. This article reviews our understanding of apoptosis in the nervous system, concentrating on its possible roles in chronic neurodegenerative disorders. **Am J Med. 2000; 108:317–330.** ©2000 by Excerpta Medica, Inc.

Apoptosis and necrosis are presently recognized as the two major types of physiologic or pathologic cell death. The term apoptosis was set forth in the early 1970s and is derived from Greek roots meaning "dropping of leaves off a tree." A number of features define apoptosis and differentiate it from necrosis, although some features are also overlapping with necrosis. A principal distinguishing factor is that apoptotic cell death is an "active" process, for which protein synthesis is required. During this process, there is consecutive activation of a variety of otherwise dormant pathways. Thus, apoptosis may be conceived of as "programmed active cell death," as opposed to "passive cell death." This distinction has led to the denotation of apoptosis as "cell suicide." Another significant feature of apoptosis is that it usually involves isolated single cells, with topographically and temporally dispersed cell death within an organ. Contrariwise, necrosis often occurs in a regional group of cells at a particular point in time.

Pathological features of apoptosis include a number of attributes morphologically and biochemically distinguishing the process from necrosis (see Table 1). Most of these are at the single cell level, although necrosis is usually a more massive phenomenon, affecting whole tissues, while apoptosis usually involves single cells, as mentioned above. Necrosis involves substantial cell *swelling*, with membrane breakdown and leakage of cell contents extracellularly. Apoptosis classically involves involutional change, with cell *shrinkage* being the characteristic shape change. During necrosis, the cytoplasmic organelles, such as mitochondria and lysosomes, usually swell and lyse, while in apoptosis there is contraction, with formation of "apoptotic" bodies, or small dense vesicular units. Apoptosis involves plasma membrane changes, clearly visualized in culture, but also recognizable in vivo, with marked cytoplasmic blebbing and involution. Nuclear changes of necrosis consist of disruption of nuclear membrane integrity; nuclear contents may show histopathological fading or intensifying, sometimes with fragmentation of the nucleus. By contrast, in apo-

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Table 1. Differences in the Pathological Features of Neural Cell Death by Apoptosis and Necrosis

	Pathological Features	
	Apoptosis	Necrosis
Pattern of death	Individual single cells	Whole groups of cells
Cell shape changes	Cell shrinkage	Cell swelling and lysis
Plasma membrane changes	Membrane preservation, cell surface blebbing	Early membrane breakdown
Organelle changes	Involution, contraction, "apoptotic bodies"	Organelle swelling and disruption
Nuclear changes	Chromatin condensation and fragmentation	Karyolysis, pyknosis (or karyorrhexis)
DNA breakdown	Internucleosomal DNA fragmentation, free 3′-ends	Diffuse and random DNA degradation
Cell degradation	Phagocytosis without cell infiltration or inflammation	Marked inflammation, with macrophage invasion

ptosis, the characteristic nuclear changes involve clumping of chromatin, sometimes leading to a "spoked wheel" appearance. A prominent molecular hallmark of apoptosis is a specific pattern of internucleosomal fragmentation of DNA. This leads to the appearance on agarose gel electrophoresis of a DNA "ladder," namely a large series of electrophoretic bands differing from their neighbors in molecular weight multiples of about 180 base pairs. In necrosis, there is also DNA breakdown, but it is apparently more diffuse and random, leading to a "smear" on gel electrophoresis rather than discrete multiple bands.

THE SETTING FOR APOPTOSIS

Normal embryonic and postnatal development includes cell death, which is generally of the apoptotic variety. As the result of either endogenous signals or intercellular signaling processes, activation of programs of cell death occurs in certain cells. These cells are developmentally superfluous either due to having already served their scaffolding or temporary function or due to cellular overproduction, faulty migration, or "erroneous" connectivity. Selective cell death is thus a standard aspect of development among multicellular species. These range from simple nematode worms and fruit flies to more complex organisms such as birds or mammals. Most commonly, death is occasioned by the presence of overexuberant production mechanisms and the subsequent need during the later phases of development for refinement of embryonic cells or structures. Such phenomena occur in a variety of different body parts of the developing organism. For example, there is a necessary deletion of clones of cells in the immune system, so as to allow self-tolerance. There are programmed losses of limb cells to yield the interdigital spaces of the limb bud, with loss of "finger webs." However, the process of developmental cell deletion is perhaps most important in the nervous system, in which highly specific connections of great accuracy are required for proper functioning of the organism, and in which behavioral experience can help in refining connectivity. Thus, during nervous system development and maturation, significant numbers of cells die at certain times and places.

These waves of cell death generally occur during periods in which cells have acquired the capacity for cell-to-cell signaling; the losses often may relate to refinement of patterns of electrical synaptic connectivity. For example, losses of nearly half the ganglion cells in the retina and the spinal motor neurons in the spinal cord may yield improvements in retinotopic and neuromuscular projections, respectively. We do not have a full understanding of the exact mechanisms by which "normal" developmental apoptosis is triggered. However, both positive, instructive stimuli, and negative, inhibitory factors have been demonstrated to be operational. Among positive factors that stimulate apoptotic cell death are corticosteroids in the lymphoid system, and various cytokines such as tumor necrosis factor alpha (TNF-α). However, it is the loss of inhibitory factors that more commonly results in activation of the apoptotic program of cell death. This has been directly demonstrated in a variety of classic experiments, many involving the dependence of sensory ganglia on nerve growth factor (NGF) support during critical developmental periods [reviewed in (1,2)], and a similar situation for spinal motor neurons during development (3). Thus, loss of cell supportive substances such as hormones or growth factors including neurotrophic substances may cause apoptosis. Conversely, however, exposure to certain endogenous hormones or factors, as well as to toxic agents or ionizing radiation, may induce apoptosis. A consequence of these observations is that it has been suggested that, in many cases, proliferation and apoptosis may often be flip sides of the same coin. Depending on environmental stimuli, cells may either proliferate or, alternatively, "commit suicide" (4).

Disease processes may be accompanied by either necrosis or apoptosis, or often both. This may depend on the intensity and extent of injurious stimuli. Thus, a variety of acute or subacute insults, including ischemia, tox-

Table 2. Mechanistic Differences between Neural Cell Death by Apoptosis and by Necrosis

	Mechanisms	
	Apoptosis	Necrosis
Causes	Developmental/programmed Degenerative changes Growth factor deprivation Mild ischemia, radiation, etc.	Toxic Massive ischemia Radiation (high dose)
Cellular processes	Programmed cascade Δ membrane phospholipid asymmetry Organelles preserved/shrunk Energy (ATP) dependence Requires protein synthesis Requires new RNA transcription	Noncoordinated events Cell membrane rupture Mitochondrial swelling Energy independence No protein synthesis No RNA synthesis
Molecular Events	Mitochondrial permeability transition Mitochondrial cytochrome c release Caspase activations Internucleosomal endonucleases Transglutaminase activation Poly(ADP-ribose) polymerase cleavage	ATP depletion Enzymatic digestion Protein denaturation Diffuse DNA digestion

ins, ionizing radiation, and tumor growth, may result in either apoptosis, particularly for low doses, or necrosis, especially at the higher doses (Table 2). For chronic degenerative disorders, the nature of the injurious signal is often unknown. And it is for these disorders that the nature of the mechanisms underlying cell losses is also less clear. For these processes, apoptosis is a logical hypothesis and not without considerable experimental support, as will be reviewed in this article. Exactly what might prompt apoptosis in the chronic disorders is unclear, but a number of factors, based in part on studies of causes of apoptosis during development, have widely been postulated as potentially etiologic. Thus, nervous system apoptosis might occur from oxidative stress that may increase with age, loss of neurotrophic (target cell factor) support, accumulated burden of endogenous or exogenous toxic factors, or excessive release of excitatory neurotransmitters known as excitotoxins (Figure 1).

CELLULAR EVENTS IN APOPTOSIS

Cytoplasmic processes that are abnormally active in apoptosis include, most importantly, a variety of proteolytic enzymes, members of the caspase family, which are usually activated as part of a proteolytic cascade in apoptosis. The actions of these enzymes destroy important cellular machinery, preventing the synthesis of new proteins and ultimately leading to irreversible injury (Figures 2 and 3). The caspase family, as presently described, consists of 10 proteolytic enzymes, named as such because they are cysteine proteases that cleave after aspartate residues. Caspases 4 and 5 have sequence homology to caspase 1, the first identified mammalian caspase, also known as interleukin 1-converting enzyme (ICE). Caspases 3, 6, 7, 9, and 10, have homology with the *ced-3* "death gene" in nematode worms. All the caspases are first synthesized as proenzymes (procaspases) that exhibit very little activity, about 1% of that of their mature form, unless highly concentrated in aggregates. Upon proteolytic cleavage into large and small subunits and further removal of an N-terminal "prodomain," they become fully active proteases. In general, caspases 8 and 9 appear to be "initiator" caspases, while caspases 3, 6, and 7 are secondarily activated and are termed "effector" caspases because they act on a variety of cell proteins. Caspases 8 and 9 may first be activated by self-aggregation. There are two alternative pathways for this activation (Table 1, Table 3). The first derives from extracellular signals and results in subplasmalemmal aggregation of caspases through an association of cytoplasmic domains of ligand receptors (eg, Fas-ligand, TNFα, or NGF "receptor" p75) and "adapter" molecules (FADD, RAIDD, and so forth). The second is mediated from within the cell, likely owing to oxidative stress involving mitochondrial injury or failure, and involves changes in mitochondrial permeability, cytosolic release of cytochrome c, association of this protein with an apoptosis-aggregating factor, APAF-1, and activation of caspase 9 (5). Regardless, the activated initiator caspases 8 and 9 then act subsequently to cleave "downstream" or effector procaspases 3, 6, 7, which in turn cleave a variety of cytosolic proteins. These susceptible proteins include structural proteins such as lamins, fodrin, and actin, DNA repair enzymes such as PARP [poly-(ADP-ribose) polymerase], and regulatory proteins such

Table 3. Selective Neural Cell Losses Occur in a Variety of Neurodegenerative Diseases

Neurodegenerative Disorders	Severely Affected Cells
Amyotrophic lateral sclerosis/ Lou Gehrig's disease (ALS)	Spinal motor neurons, corticospinal (layer 5) Betz cells
Parkinson's disease (PD)	Substantia nigra pars compacta, locus ceruleus, vagus dorsal motor nucleus, sympathetic ganglia
Huntington's disease (HD)	Caudo-putamen medium spiny interneurons, cortical layers 3, 5, and 6 neurons
Olivopontocerebellar atrophy/ spinocerebellar ataxia type 1 (SCA-1)	Cerebellar Purkinje cells, dentate nucleus, inferior olive
Machado-Joseph disease/ spinocerebellar ataxia type 3 (MJD1/SCA-3)	Dentate nucleus, red nucleus, substantia nigra pars compacta, Purkinje cells, brainstem motor nuclei
Alzheimer's disease (AD)	Entorhinal neurons, hippocampal neurons, cortical neurons (late in disease)

as nuclease inhibitors (ICAD), whose cleavage leads to nuclease activation (5,6). Thus, the caspases constitute key elements of a proteolytic cascade, acting in similar fashion to the factors involved in the blood coagulation or complement cascades (Figures 2 and 3).

A variety of cellular molecules appear to interact with the caspase cascade or in other parts of the apoptotic pathway (5–7). These include a family of "inhibitors of apoptosis" including NAIP, X-chromosomal IAP, human IAP-1 and IAP-2, crmA, and p35. Many of these act as protease inhibitors, directly decreasing the activities of different caspases. Another family of apoptosis-regulating proteins is the Bcl-2 family, which are homologous to the *C. elegans* cell death suppressing protein ced-9. Many of this family (Bcl-2, Mcl-1, Bcl-w, Bcl-xL) do generally act to inhibit apoptosis. However, other family members (Bax, Bcl-xS, Hrk, Bak, Bid, Bik, Bad) seem to inhibit cell survival during apoptosis and thus are proapoptotic. These different proteins may be associated with mitochondria and thus relate to initiation of apoptosis through mitochondrial stress. For example, Bcl-2 apparently decreases release of cytochrome c (Figure 3). A host of other proteins, including oncogene products, many of which are important for the cell cycle (including p53, myc, and rpr), and others likely important for their interactions with caspases (including Apaf-1 and cytochrome c) promote apoptosis or in some cases inhibit the induced pathways.

Nuclear changes, considered a hallmark of apoptosis, consist of nuclear fragmentation from chromatin condensation and internucleosomal DNA breakdown. Indeed, DNA laddering is often used as a diagnostic sign of apoptosis. DNA degradation is accomplished by abnormally activated endonucleases. A number of such activities, mostly divalent cation-dependent enzymes, have been identified as candidates, including DNase I, DNase II, Mg2+-dependent endonuclease AN34, DNase gamma, and Nuc70 (8,9). However, the caspase-activated DNase/DNA fragmentation factor CAD/DFF40 (10), likely the homolog of CPAN (11), with the inhibitor chaperone known as ICAD/DFF45, seems the best characterized. Caspase-cleavage of the inhibitory ICAD results in release of the CAD nuclease, degradation of chromosomal DNA in a ladder pattern, chromatin clumping, and irreversible nuclear destruction (10–16).

Cell surface changes are also prominent in apoptosis.

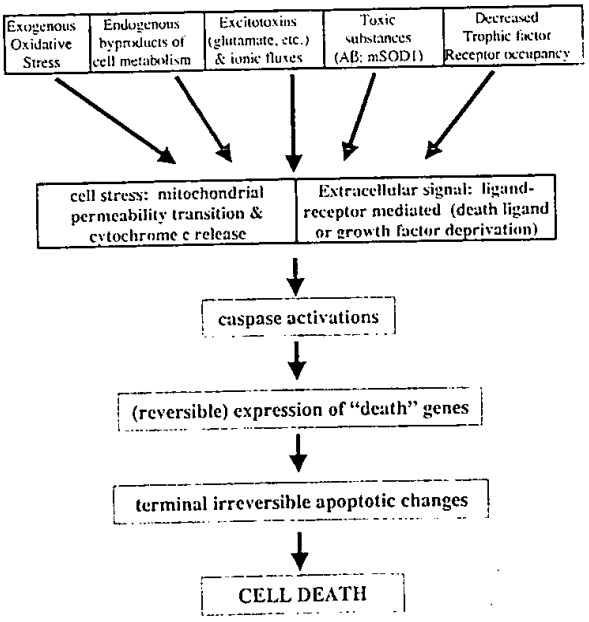

Figure 1. Presumed causes and general scheme of apoptosis in neurodegeneration.

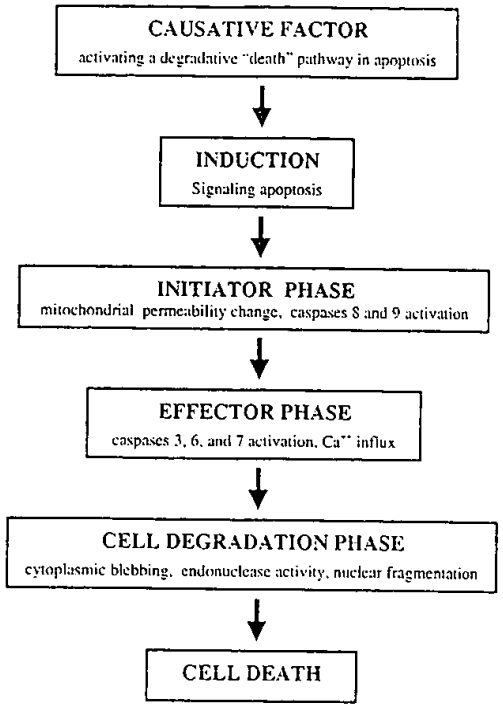

Figure 2. Pathway of deleterious events in apoptosis.

Although plasma membrane integrity is initially preserved, notable membrane blebbing occurs, with eventual loss of plasma membrane integrity. During apoptosis, there is "flipping" of the phosphatidylserine lipids in the membrane from their usual predominance in the internal portion of the bilayer to an external location. Cell membrane changes are not only a consequence of activation of the proteolytic cascade of apoptosis, but also can accelerate the process. This results from intracellular calcium, sodium, or potassium influxes, which can aggravate the cascade of deleterious proteolytic, phospholipase, and endonuclease enzymes.

FACTORS THAT MAY CAUSE OR INDUCE APOPTOSIS

Growth factor deficiencies prompt apoptosis in the developing nervous system. Thus it is natural to suspect that loss of retrogradely transported neurotrophic factors might be important in causing programmed cell death in adult neurons. Oxidative stress is a presumptive cause of apoptosis. In particular the relationship of mitochondrial failure to apoptosis is strong. A known signal for apoptosis is change in mitochondrial permeability, leading to release of cytochrome c into the cytoplasmic compartment (Figure 3). Excitotoxic neuronal injury may be a precipitating factor in apoptosis (Figure 1). Excessive levels of glutamate result in cell depolarization, influx of Ca^{++} ions, and activation of caspases and the apoptotic cascade.

Neuronal Cell Loss in Neurological Disease

Primary versus secondary cell losses. Cell loss in disease may occur as an early and key feature of a disease process or may occur secondarily, relatively late in disease. Neurons are evidently the most complicated cells in the body morphologically. Most neurons have a dendritic arbor, cell soma, and an axon with its terminal ramification. Synapses are the connections between neurons. Thus, injury to neurons in the nervous system can occur at the level of the synapse, the axon nerve terminal, the axon fiber, the myelin sheath of the axon fiber, the axon hillock, the dendrites, or the cell body (soma) or any of its organelles, namely nucleus, mitochondria, endoplasmic reticulum, Golgi apparatus, and so forth. Furthermore, injury to neurons may occur secondarily because of injury to supporting cells including astrocytes, microglia, oligodendroglia, or vascular elements. Ultimately, injuries peripheral to the cell body, for example at the level of the synapse or axon, may still cause neuronal cell injury and death.

Cell loss in acute neurological conditions. Stroke, trauma, and infection cause severe, usually focal injuries to the central nervous system. In general, such severe cataclysmic injuries due to hypoxic or ischemic insult to the brain cause necrosis, although in most cases there is a process of delayed "secondary" injury in a "penumbra"

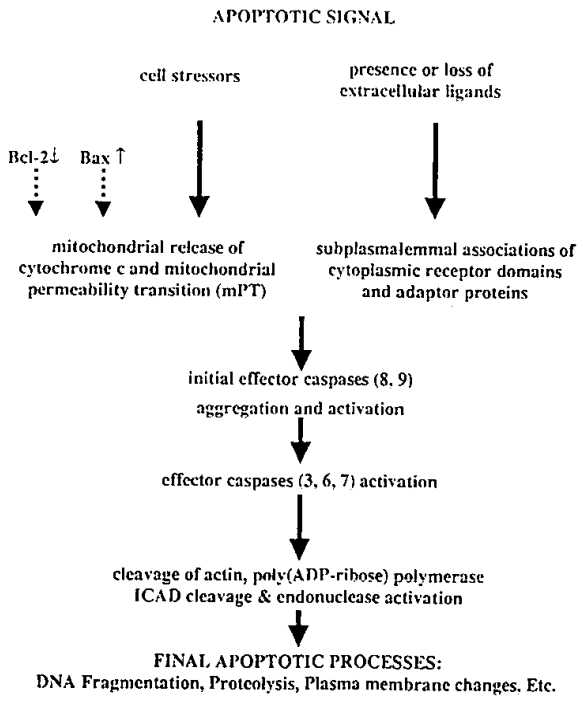

Figure 3. Molecular elements of apoptotic cascade.

Table 4. Detection of Apoptosis*

Basis of Method	Specific Techniques
DNA ladder	TUNEL ISEL
Chromatin clumping	Bisbenzimide (Hoechst 33258) stain, propidium iodide stain, acridine orange stain
Light microscopy	Membrane blebbing, cell shrinkage
Electron microscopy	Cell fragmentation, chromatin clumping
Immunochemistry	Apoptosis-specific protein, c-jun, c-fos, c-myc, P53, HSP70, bcl-2, etc.
Western blot	Poly(ADP-ribose)polymerase (PARP) cleavage, caspase-3 cleavage
Experimental inhibition	Cycloheximide, actinomycin D

* Methods used to detect apoptosis include a variety of techniques of various specificities.
TUNEL = TdT-mediated dUTP nick end labeling, detects free 3'-OH DNA ends; ISEL = in situ end labeling, uses *Eco* DNA Pol Klenow fragment to label double strand DNA breaks.

zone, initially spared and surrounding an area of most severe damage. Some of the cell death in these penumbra areas appears to be caspase-mediated and may be more of an apoptotic type (17–23). This may not be evidence for different insults in the penumbra, or at-risk zone, but rather may be evidence for a general principle that qualitative cell insults of a type that cause necrosis, when delivered at a "subnecrotic" level, may instead cause apoptosis (24,25). Thus, a variety of acute neurological diseases may, in part, involve apoptotic death. However, this review will focus on cell loss in chronic neurological disease.

Cell loss in chronic neurodegenerative conditions. Neurodegenerative diseases include a variety of progressive disorders of the central nervous system, resulting in cognitive and/or motor deterioration. These disorders have classically been distinguished by their clinical symptoms, which in turn, have generally been well-correlated with the observed neuropathology. These disorders are thus often marked by a predominant pattern of involvement affecting one or another portion of the central nervous system (see Table 3). Hence, a number of these disorders are known for primarily affecting motor systems, and may only rarely, late in the disease, mildly affect cognition. These include amyotrophic lateral sclerosis (ALS), Parkinson's disease, Huntington's disease, spinobulbar atrophy, and the spinocerebellar ataxias. The pathoetiology of these disorders is predicated upon involvement of spinal motor neurons, corticospinal neurons, striatal neurons, or cerebellar nerve cells. Another set of disorders is known for primarily affecting cognition and only secondarily affecting motor performance. These include the most common neurodegenerative disorder, Alzheimer's disease, and certain related disorders, among which are Pick's disease, now known to be one of the frontotemporal dementias.

Neurodegenerative conditions may also be distinguished by the cytologic localization of the site of injury. In ALS, Parkinson's disease, Huntington's disease, and some of the spinocerebellar ataxias (eg, olivopontocerebellar ataxia/spinocerebellar ataxia type 1 [SCA1], Friedreich's ataxia, and Machado-Joseph disease/spinocerebellar ataxia type 3 [MJD/SCA3]), losses of neuronal cell populations are an early feature of the disease; they seem key to disease pathogenesis. In these disorders, cell loss is apparently a primary feature of the disorder, and changes in axons and synapses are secondary to the neuronal injury. For such diseases, in which cell death is an apparently primary feature of the disorder, it is clear that apoptotic mechanisms must be considered likely candidates for the mechanism of cell death. For other neurodegenerative conditions, notably Alzheimer's disease and the frontotemporal dementias, it is less clear that neuronal cell losses are early features of the neuropathological tableaux. In fact, accumulations of intraneuronal inclusions, the tangles, extraneuronal protein deposits (amyloid-containing senile plaques in Alzheimer's disease), and synaptic losses appear to be the earliest accompaniments of disease symptoms. Although selective cell losses do ensue later in the course of the disease, it is less clear as to whether these may be primary effects of the disease or, for example, secondary results of synaptic injury. Irrespective of the primacy or derivative nature of cell death in Alzheimer's disease, the mechanism of cell demise is unknown. It has been suggested that a prominent mechanism may be apoptosis. A variety of techniques are available for assessment of apoptosis (Table 4), although DNA

fragmentation assays (TUNEL, ISEL) are perhaps most widely used.

SPECIFIC DISORDERS

Amyotrophic Lateral Sclerosis

Loss of motor neurons is a hallmark of amyotrophic lateral sclerosis, a relatively rapidly progressive disease in which spinal motor neurons and upper motor neuron pathways degenerate, leading ultimately to complete paralysis of all general somatic musculature. Death usually occurs within 3 years from respiratory insufficiency or its complications. ALS is also known as motor neuron disease in Great Britain. In the United States, it is often referred to as Lou Gehrig's disease, after the famous baseball player stricken in his prime by this disease. The disease is of sporadic onset in 95% of patients, although there are some familial cases. Somewhat less than one third of the familial cases have a mutation in the cytosolic Cu-Zn superoxide dismutase (SOD1). The pathological hallmarks of both the sporadic and familial forms of the disease are loss of anterior horn cells (lower motor neurons) and loss of Betz cells, the projecting upper motor neurons, in the motor cortex, with corresponding changes in the corticospinal tract. Typically, early in the disease, only a single limb or pair of limbs may be affected; with progression, there is increasing involvement of the contralateral limb and adjacent ipsilateral extremity with concomitant progressive bulbar involvement, affecting speech and swallowing. Overall, the disease is often first noted from its lower motor neuron involvement, with typical muscle atrophy, fasciculations, and flaccid weakness. Although the balance of upper and lower motor neuron signs and symptoms varies on a patient-to-patient basis, cell losses are essentially confined to motor neurons.

There are a several variants of ALS, including progressive bulbar palsy, in which the principal involvement is bulbar, rather than spinal; progressive muscular atrophy and spinomuscular atrophy (in children), in which involvement is exclusively at the level of the spinal motor neurons, with no detectable upper motor neuron involvement; primary lateral sclerosis, a rare, progressive pure upper motor neuron syndrome, differing from ALS in the lack of involvement of spinal motor neurons; and X-linked spinobulbar atrophy, also known as Kennedy's syndrome, which is one of the genetic "triplet" disorders.

A unifying feature of the pathologies of the motor neuron diseases mentioned above is rather selective loss of motor neurons over time. This is especially evident on pathological examination at death, whether ultimately from the disease or in some cases from untimely premature circumstances. Unfortunately, there is no in vivo, noninvasive tool that has excellent performance at assessing motor neuron health or number. However, electrodiagnostic testing is able to demonstrate the loss of motor neuron innervation of muscle over time, and the concomitant, presumably compensatory, sprouting of surviving axons to innervate denervated muscles. All the evidence by pathological, clinical, and laboratory examinations indicates motor neuron cell loss as a primary, and rather specific, element of this disease. Thus, considerable effort has been expended in furthering understanding of the reasons for the specificity of this process for motor neurons and the mechanisms of cell death for these motor neurons. Furthermore, it is well known that motor neuron death occurs developmentally during a period of refinement of neuromuscular connectivity, and that motor neuron death, demonstrably apoptotic, occurs in neonatal animals in response to deprivation of growth factors and can be ameliorated by replacement factor therapy. Therefore, it is not unreasonable to hypothesize that the motor neuron cell death that occurs in ALS is due to apoptosis.

Possible causes of ALS include peripheral deficiencies of growth factors leading to retrograde degeneration, oxidative stress in motor neurons, and glutamate excitotoxic injury to motor neurons. Each of these factors might likely operate through induction of apoptosis (Figure 1). For ALS, as for other degenerative disorders of the nervous system, mouse model systems may be used for investigation. These fall into several categories. First, there are naturally occurring or induced mutations that seem to cause motor neuron degeneration syndromes (eg, pmn, mnd, wobbler). Second, transgenic mouse models based on the expression of known human familial ALS mutations in the SOD1 gene show motor neuron degeneration much like affected humans. Third, certain other transgenics, such as interleukin-3, or neurofilament overexpressers or underexpressers may have a phenotype that resembles human ALS. In some of these systems, there is the suggestion, at least terminally, of apoptotic degeneration, and this is discussed below.

Studies of human autopsy tissue show marked depletion of motor neurons. Examination of ALS spinal cord has shown evidence for apoptosis by TUNEL stain (26). Supportive studies of apoptotic related proteins have revealed decreased antiapoptotic Bcl-2 mRNA and increased proapoptotic Bax mRNA in spinal neurons (27). Although others have found immunochemically demonstrated increased Bcl-2 in brain and spinal cord of ALS patients (26,28), most evidence suggesting a role for apoptosis in ALS involves study of the SOD1 protein using in vitro and mouse models. While transfection of neural cells with wildtype SOD1 appears to be antiapoptotic, mutant SOD1 expression increases apoptosis (29,30). Mutant SOD1 (A4V and V148G) caused death of transfected cells, which could be prevented with Bcl-2, antioxidants and caspase-inhibitors (31); and mutant SOD1 has

been shown to activate caspase 1 (32). Overexpression of the antiapoptotic protein Bcl-2 apparently prolongs SOD1 mutant mice survival (33). However, Bcl-2 transgenes may result in greater absolute numbers of motor neurons developing in the spinal cord. Other antiapoptotic reagents also increase survival of SOD1 mutant mice. Mutant caspase 1 (C285G) acts as a dominant negative inhibitor of caspase 1/ICE. Whether microinjected into wild type dorsal root ganglion cells or transgenically engineered into mice, the expressing sensory neurons show reduced apoptosis upon withdrawal of neurotrophic factor (34). The benzothiazole drug riluzole mildly prolongs the course of ALS in humans (35–37) and apparently acts similarly in SOD1 mutant mice, prolonging time to death (38). However, its putative mechanisms of action are likely upstream of any apoptosis per se, and include inhibition of glutamate release and interference with glutamatergic intracellular signal transmission (37). Thus it is possible that riluzole acts to prolong the course of motor neuron disease by decreasing glutamate-associated neuronal cell death, which may be occurring by apoptosis. Vitamin E (α-tocopherol) is another medication postulated to prolong the course of motor neuron disease perhaps due to alleviation of oxidative stress (38–40). Although vitamin E is of no proven utility in human ALS, it does appear to slightly prolong the course of disease in the murine mutant SOD1 model (38).

Spinal muscular atrophy is the term for a set of inherited autosomal recessive motor neuron diseases, which involve degeneration of the anterior horn cells or lower motor neurons, and typically have onset before the age of 21. Two closely neighboring genes on chromosome 5q, *SMN* and *NAIP*, appear to be involved. The *SMN* gene codes for an element of the spliceosomal complex. The *NAIP* gene product is one of the family of neuronal apoptosis inhibitory proteins (41–43), referred to above, and inhibits apoptosis from a variety of causes (42). Hence, complete deficiency of this protein, due to the homozygous mutant state, might lead to increased motor neuron apoptosis and motor neuron degeneration, causing spinal muscular atrophy.

Parkinson's Disease

Parkinson's disease is a degenerative disease of the extrapyramidal motor system. Specifically, failure of the nigrostriatal system, due to cell loss in the substantia nigra, results in the classic symptoms of resting tremor, rigidity, bradykinesia, and postural instability. The disease progresses slowly over decades. Initially, there is only mild-to-moderate nigral cell loss, and the disease is quite amenable to neurotransmitter "replacement" or "replacement equivalent" therapies with levodopa or dopaminergic agonists. However, ultimately, the disease becomes refractory to therapy, apparently because of the severity of nigral cell losses and secondary striatal changes. This leads to severe motor incapacitation, not infrequently also accompanied by cognitive deterioration.

Selective losses of neurons in the substantia nigra and other deep nuclei are characteristic of Parkinson's disease. The most prominent pathological finding of Parkinson's disease is loss of the normally obvious pigmented cells in the pars compacta of the substantia nigra in the ventral midbrain. These cells are responsible for dopaminergic modulation of the cortico-striato-thalamo-cortical motor loop. It is this marked predominance of disease in one cell type, the substantia nigra neuron, that allows replacement therapy to be so successful. However, there are also notable selective losses of cells elsewhere in the nervous system, some of which may lead to clinically relevant deficits. For example, the locus ceruleus, with its associated noradrenergic output, and the dorsal motor nucleus of the vagus are also affected by cell losses in Parkinson's disease. Nonetheless, the very selective nature of the gradual cell losses in Parkinson's disease has given rise to the hypothesis that these cells might die as a consequence of apoptosis.

Parkinson's disease pathologically involves not only prominent substantia nigra cell losses as described above, but also shows a pathological hallmark, the Lewy body. This is a pale eosinophilic neuronal cytoplasmic inclusion body, now known to consist of α-synuclein and ubiquitin. Lewy bodies are present in the substantia nigra and also are often present more widely, with a distribution encompassing other brainstem nuclei such as the locus ceruleus and dorsal motor nucleus of the vagus, and even the peripheral nervous system (eg, sympathetic ganglia) and areas of the cerebral cortex (eg, they are found particularly often in cingulate cortex). A key question is whether these inclusion bodies are simply an index of injured or dysfunctional cells, destined to die, or whether these inclusions might themselves cause neuronal cell death. Whether these inclusions are cause, or effect, of a cell death program, it is not proven whether the cell death that clearly does occur in Parkinson's disease is by means of an apoptotic mechanism.

Evidence for apoptosis in Parkinson's disease is present in human tissue and animal models. Studies examining the substantia nigra pars compacta (SNpc) in brains of humans with Parkinson's disease have in some cases shown signs of neuronal apoptosis (44–46). However, other results have been negative or unconvincing (47–51). Investigation of apoptotic proteins in Parkinson's has shown elevation of antiapoptotic Bcl-2 in the patients' striatum, consistent perhaps with a reactive role (52). Examination of mice rendered parkinsonian by means of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment has shown evidence for apoptosis in the substantia nigra (53). However, this model system clearly involves oxidative injury (exposure to the MPP+

derivative of MPTP), which has been shown to cause apoptosis also in cultured cells (54). The pathoetiology of idiopathic Parkinson's disease in humans may or may not involve related oxidative stress. Concern has been raised that levodopa, the effective treatment for Parkinson's disease, can induce apoptosis in cultured neural cells (55, 56); however, there is little evidence that this effect applies to treated humans (57). Interestingly, selegiline, another drug used to treat Parkinson's, has been shown to have an antiapoptotic role in cell culture (58,59), although these effects have also not been proven to be clinically relevant.

Possible causes for the neuronal losses in PD include abnormal deposits of the protein α-synuclein, the major constituent of Lewy bodies, oxidative stress, loss of target (striatum)-derived growth factors such as GDNF, and excitotoxicity injury. It is now known that Parkinson's disease can arise from a genetic mutation in the α-synuclein gene; and in fact, aggregates of mutant A53T α-synuclein have been reported to cause apoptosis in cultured neuroblastoma cells (60). Nonetheless, the vast majority of Parkinson's disease is sporadic, and without a demonstrable genetic component, and it is not clear whether the synuclein deposits are cause or consequence of the disease. The idea that oxidative stress might cause apoptotic injury to the substantia nigra neurons is also attractive, since these cells are especially replete in oxidative enzymes used in the synthesis of levodopa.

Huntington's Disease

Selective cell losses in the basal ganglia are the pathologic hallmarks of Huntington's disease. The classic appearance of caudate atrophy is a function of these neuronal losses. Apoptosis, as assayed by TUNEL staining, has been observed in Huntington's disease striatum (48,61). Huntington's disease is caused by a CAG triplet repeat in the gene *huntingtin* on chromosome 4. The CAG triplet causes expression of a polyglutamine tract that is also capable of causing protein aggegation. The *huntingtin* knockout mouse is embryonic lethal, and the abortive embryos seem to show considerable apoptosis (62), perhaps implying that a role of *huntingtin* is to suppress apoptosis. Presumably the mutant form might be less antiapoptotic even in adults. Recently it has been shown that caspases, including caspase 3, cleave *huntingtin* (63–65). The mutant form of the protein, with more CAG repeats, is cleaved at higher efficiency than the normal form and is associated with increased vulnerability to apoptosis (65,66).

Spinocerebellar Ataxias

Selective neuronal losses occur in the cerebellum and brainstem in this group of autosomal dominant inherited ataxias, now numbered spinocerebellar ataxia type 1 (SCA1) through SCA10, and the related condition DRPLA (dentatorubropallidoluysian atrophy). Selective chronic neurodegenerative loss of Purkinje and/or other cells occurs during these diseases. Most of these diseases are known to be triplet-repeat disorders, like Huntington's disease, in which abnormal expansion of a region of CAG repeats (coding for polyglutamine) is associated with disease. It has been reported that ataxin-3 (coded for by the SCA3/MJD1 gene) and atrophin-1 (coded for by the DRPLA gene) are both cleaved by caspases (64). It can be speculated that the associated accumulation of polyglutamine rich fragments contributes to the pathogenesis of the disease. A recent report suggests the expanded genes are themselves apoptotic. Cultured cells undergo apoptosis when transformed with a cDNA coding for a portion of the SCA3/MJD1 gene that includes the abnormally expanded CAG repeats (67). Furthermore, in a *Drosophila* model, transfection with DNA coding for the antiapoptotic viral P35 protein resulted in partial protection from the late-onset cellular degeneration caused by expression of the abnormal MJD partial protein (68).

Alzheimer's Disease

Alzheimer's disease is the most common cause of intellectual deterioration in modern-day society. Estimates are that about 2% of the population, or about 4 million individuals, are affected in the United States. The incidence increases markedly with age. Population based surveys suggest that in the population over age 85 years as many as 50% to 60% of individuals may be afflicted with the disease. Pathologically, the hallmarks of Alzheimer's disease include senile plaques, consisting of extracellular depositions of β-amyloid peptide, and neurofibrillary tangles, which are intracellular depositions of the protein tau, in a hyperphosphorylated and ubiquitinated state.

Selective neuronal losses occur, especially in particular regions of the hippocampus. There are also profound losses of neocortical synapses, possibly related in part to neuronal losses, in addition to the tau and amyloid protein deposits described above. The exact relationship between losses of cells and the pathogenetic cascade of the disease is unclear. As discussed above, the cell losses could be a primary part of the disease process or could be the secondary results of cell injury at levels more distal to the cell body, such as at the synapse, dendritic arbor, or even axon. However, whether primary or secondary, these selective cell losses have particularly prompted consideration of the hypothesis that apoptosis may be responsible for cell disappearance and may possibly play a key role at the root of the disease process.

Recent advances in the molecular genetics of Alzheimer's disease have resulted in the identification of four specific genes that are proven to be involved in the occurrence of this disorder. Three of these genes code for membrane spanning protein genes, β-amyloid precursor protein (βAPP), presenilin-1 (PS1), and presenilin-2 (PS2), mutations of which together are responsible for a significant fraction of autosomally dominant inherited early-

onset Alzheimer disease. A large variety of point mutations in PS1 (about 50) and PS2 have been found that, when present, obligatorily lead to signs and symptoms of the disease commonly prior to the usual onset age of 50 to 70 years (depending upon which allele of which gene is affected). The gene products of the βAPP, PS1 and PS2 genes are thus certainly involved in the pathogenesis of Alzheimer's disease in these families and very likely involved in sporadic Alzheimer's disease. Specific genotype for the fourth gene, apolipoprotein E, which is polymorphic in the general population, with three common alleles (known as 2, 3, and 4), affects the relative risk of developing late-onset Alzheimer's disease in the general population, but is not a deterministic factor. The identification of these genes has provided marked advances in the understanding of Alzheimer's disease pathways. For example, it is now evident that all the mutant or unfavorable forms of all these genes results in increased β-amyloid deposition in the brain. And it is widely hypothesized that extracellular β-amyloid may have an apoptotic influence on cells. It might be through such an influence that cell death occurs in Alzheimer's disease. In addition, studies on the nature of PS1 and PS2 have benefited greatly from research on lower animals, namely the invertebrate nematode worm and fruit fly, which have shed light on the cellular functions of these genes. Of great interest is the observation that regulation of apoptosis may be an important function of the presenilins. Some evidence suggests that these serpentine transmembrane proteins might be antiapoptotic, and that mutations might thus operate by increasing apoptosis.

Apoptosis in the Alzheimer disease brain may be responsible for the neuronal cell losses that are features of Alzheimer's disease. The brains of deceased individuals with Alzheimer's disease have been examined for evidence as to the nature of the processes leading to the depletion of cell populations. However, this is limited by the fact that cell loss is a dynamic process that almost certainly occurs over some years. Neuropathologic examination, usually by autopsy, but occasionally at incidental or diagnostic biopsy, represents only a "snapshot in time." Thus, neuropathologic studies may not have adequate sensitivity to detect relatively rapid processes, such as apoptosis. Simple calculations are useful. Apoptosis often occurs from start to finish in 2 to 6 hours, but a conservative estimate for the duration of visible signs of an apoptotic cell might be 24 hours (1 day). Generally, there may be only a 20% to 80% reduction in cell number in a given region over a period of about 10 to 20 years of disease. Here we might assume a 40% reduction over approximately 4,000 days, Thus, it is likely that only about 40% × (1/4,000) of cells, or about 0.01% of cells, might be expected to manifest ongoing apoptosis in a given, temporally-static, histologic specimen.

Despite the above analysis, there have been a number of reports by investigators finding increases in markers of apoptosis in brain specimens from autopsies of Alzheimer's disease patients (69–75). Some investigators have indeed only found rare neurons in brain specimens showing markers of apoptosis (72). However, other investigators (70,75) have found widespread evidence for "apoptosis" in the entorhinal cortex of Alzheimer's disease patients, an area ultimately undergoing considerable involutional changes in Alzheimer's disease. These latter studies have indicated that as many as 80% of neuronal nuclei show TdT labeling by the TUNEL technique. However, it is less clear that this represents definite apoptotic activity, since such a high proportion of affected cells would necessarily lead to rapid and extreme depletion of cells, greater than that observed. Also, confirmation of apoptosis in Alzheimer's brains by use of gels designed to display DNA laddering have generally failed to find evidence confirming significant internucleosomal DNA degradation as an explanation for the TdT labeling. Indeed, it has been suggested that the observed TdT labeling is not indicative of apoptosis (76) when the results of an impaneled collection of immunochemical markers are considered. Nonetheless, it has been argued that the TdT labeling reflects minor DNA damage and repair that might imply an apoptotic tendency (75). Supporting this view, it has been found that expression of both the antiapoptotic proteins Bcl-2 and the proapoptotic protein Bax may be upregulated (70,77) in areas of the brain showing increased TUNEL staining and ultimately afflicted by considerable cell death (eg, entorhinal cortex). Such changes might possibly be suggestive of a fine apoptotic balance, with compensatory attempts to undergo or "stave-off" apoptosis. However, other investigators find increased proapoptotic Bax, without antiapoptotic Bcl-2 neuronal staining (78), and increased Bax (79), with cautionary warnings regarding antibody-staining variability. Recent reverse transcription polymerase chain reaction (RT-PCR) investigation of a whole host of apoptotic-related protein mRNAs revealed only increased transcription of caspase 1 in Alzheimer's brains (80).

β-Amyloid induces cell death. A key neuropathologic feature of Alzheimer's disease is the abnormal deposition in extracellular brain plaques of β-amyloid (Aβ) protein, a 39 to 42 amino acid, relatively insoluble peptide. Thus, it is natural that considerable efforts have been devoted to understanding the role of Aβ in the brain. The β-amyloid protein arises through the proteolytic degradation of a precursor protein, through the action of two putative protease activities, β-secretase and γ-secretase, upon the β-amyloid precursor protein (βAPP or APP), a transmembrane protein whose normal cellular function is still

unclear. A large literature exists on examining the effects in vitro of the β-amyloid protein in cultured cell systems. There may actually be some "trophic," or beneficial effects, of this peptide on cell survival when it is utilized at very low concentrations (81). However, at higher concentrations, particularly in preparations in which the Aβ protein has been allowed to self-aggregate, extracellular application of the protein results in apoptotic cell changes (75,82–87). These occur in a variety of cell types including PC12 cells, neuroblastoma cells, and rodent cortical and hippocampal neurons. The evidence for apoptosis includes an "imploding cell" appearance, cell shrinkage with membrane blebbing, and DNA-laddering demonstrable at the molecular level. In general, control experiments using scrambled or reverse peptides of the same amino acid content and length as Aβ have not shown similar effects. However, it is not universally agreed upon that Aβ toxicity is via apoptotic, rather than necrotic, action; some studies have found early release of lactate dehydrogenase from Aβ-treated PC 12 cells, suggestive of early loss of plasmalemmal integrity, with morphologic findings of cell swelling and lysis also more consistent with necrotic cell death (88).

The ability of the APP precursor protein, from which Aβ is generated, to cause cell apoptosis when expressed endogenously has also been investigated. The 695 amino acid form of the normal precursor protein, APP695 wt, expressed in COS cells reportedly causes increased apoptosis (89–92). It has further been reported that expression in COS cells of mutant APP V642 causes DNA breaks and apoptosis (90,91,93,94). This APP V642 induced apoptosis is inhibited by pertussis toxin (91), suggesting that the apoptosis might be mediated via a G-protein signaling pathway, inhibited by pertussis toxin (G_i or G_o). It is not certain whether APP induction of apoptosis might be through changes in formation of Aβ or, more likely, through a separate signaling pathway, and whether this is relevant to the processes occurring in Alzheimer's disease, in which Aβ production is most likely the most important factor. The mechanism of Aβ toxicity itself may be via excitotoxicity (95), via competition for the low-affinity nerve growth factor receptor, LA-NGFR/p75 (96), or via stimulation of free radical formation and oxidative stress (97–99). In vivo studies on produced transgenic mice have been reported to show that mutant APP transgenes, such as the platelet-derived growth factor promoter-driven human APP V717F mice, result in the presence of apoptotic cells in the brain (100).

Presenilins may relate to neuronal apoptosis. Presenilins, mutations of which cause Alzheimer's disease, may also relate to neuronal apoptosis. PS1 mutations may increase Aβ production, increasing apoptosis upon exposure of cells to Aβ. PS1 mutants may also increase Ca++ (owing to effects on the endoplasmic reticulum), increasing apoptosis (85,101). Transfection of PC12 cells by PS1 mutant L286V cDNA increases apoptosis in response to Aβ or NGF deprivation (102–104). Mutant PS1 expression increases expression of Par-4 (a putatively proapoptotic protein originally identified in prostate cancer cells undergoing apoptosis), mitochondrial dysfunction, and apoptosis (105). Mutant PS1-induced apoptosis has been reported to be prevented by treatment with vitamin E, possibly as an antioxidant, the calcium channel blocker nifedipine, and dantrolene, which blocks membrane-stored Ca++ release. Both normal wild type PS1, and mutant PS1 A246E have been reported not to increase apoptosis from etoposide or staurosporine (92). PS1 is cleaved during apoptosis (106). If apoptosis is induced in U937 cells by p53 and p21, then PS1 expression declines (107). Thus, there is evidence that PS1 may be involved not only as an actor in the apoptotic pathway of stressed cells, whether intrinsically or through effects on Aβ, but also that PS1 itself is a target of the proteolytic enzymes terminally induced by the apoptotic pathway.

Like presenilin 1, presenilin 2 (PS2) mutations are responsible for a fraction of early-onset familial Alzheimer disease. The gene is highly homologous to PS1, about 70% at the amino acid level, and like PS1 is a membrane-spanning protein. In similar fashion to the data for PS1, studies suggest that PS2 may be involved in apoptosis in the nervous system. Humans with mutant PS2 have increased Aβ deposition in the brain, and their cells form a greater proportion of Aβ1–42 in culture. It is likely that PS2 localizes to the endoplasmic reticulum or Golgi apparatus. It is reasonable to suppose that PS2, like PS1, may act by affecting the processing of APP. This could result in more Aβ, and thus more Aβ-induced injury, perhaps by apoptosis. However, there is also evidence that the PS2 gene product itself might have a direct role in apoptosis. PS2 has significant sequence homology with a mouse gene, ALG-3, and expression of this protein is able to protect against fas-ligand induced apoptosis in T-cell hybridoma 3DO cells (108). Mutant PS2 introduced into HeLa cells by transfection increases spontaneous cell apoptosis (109). Transfection of PC12 cells by mutant PS2 increases apoptosis induced by Aβ, nerve growth factor withdrawal, staurosporine, or the oxidative chemical, hydrogen peroxide (110,111). If these apoptosis-prone PC12 cells expressing mutant PS2 were transfected by antisense PS2 oligodeoxynucleotide, there was protection against the increased apoptosis (111); pertussis toxin also inhibited the induced apoptosis, presumably through inhibition of a G-protein–mediated signaling pathway. Just as PS1 is cleaved during apoptosis, normal wild type PS2 is also cleaved during apoptosis. Furthermore, mutant forms of PS2, such as 141N, are cleaved more efficiently

(106). As for PS1, the significance of PS2 being a target during apoptotic protein cleavage is not clear.

SUMMARY

Apoptotic cell death likely occurs in a spectrum of neurologic disorders. In many chronic neurodegenerative diseases, it is likely that cell death does play a primary role in loss of function, although this is not proven. For example, it remains a strong possibility that synaptic injury precedes neuronal injury in Alzheimer's disease. Assuming a primary, or even secondary, role for cell death in degenerating nervous system diseases, there is suggestive evidence for a variety of conditions, mostly based on in vitro and model systems, that some of such death occurs via apoptosis. During apoptotic cell death, there is activation of complicated interlocking sets of pathways, involving cytosolic, organelle, cell surface, and nuclear cellular elements. Whether intervening in the cascade of programmed cell death, or even totally halting apoptosis, would have any significant impact on the signs or symptoms of disease is currently unknown. It may be that apoptotic death is itself too late a phenomenon, and that the signals or stresses leading to neuronal apoptosis are themselves the earlier cause of irreversible nervous system dysfunction. However, continued investigations into the cell pathways involved in programmed cell demise can only increase our understanding of cell death in human development, health, and disease.

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